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Cloning of hemoglobin-α1 from half-smooth tongue sole (*Cynoglossus semilaevis*) and its expression under short-term hypoxia

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Abstract: This study cloned the hemoglobin α1 from the marine teleost, the half-smooth tongue sole (*Cynoglossus semilaevis*), and then examined its expression under hypoxia exposure. The full-length of CsHb-α1 (594 bp) cDNA contains an open reading frame encoding 144 amino acids. Sequence analysis shows that the predicted CsHb-α1 amino acids shares high identities with that of other species. Real-time PCR showed that CsHb-α1 was highly expressed in the heart, liver, spleen, kidney and blood. Five to 120 min esposure and long-term (36 h) exposure to hypoxia (1.0 mg/L) significantly increased CsHb-α1 mRNA expression in most tissues compared to those fish held in normoxic conditions (dissolved oxygen (DO): 6.2 mg/L). These results suggested that the up-regulation of Hb-α1 is an important component for adaptation of half-smooth tongue sole to short-term hypoxia.

Key words: *Cynoglossus semilaevis*; Hemoglobin; Gene clone; Hypoxia stress

半滑舌鳎血红蛋白 α1 的基因克隆及其在短期低氧胁迫下的表达分析

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摘要：该文克隆了海洋鱼类——半滑舌鳎（*Cynoglossus semilaevis*, Cs）的血红蛋白 α1（CsHb-α1）基因全长 cDNA 序列，并研究了其在短期低氧胁迫下的表达变化。CsHb-α1 cDNA 全长 594 bp，编码 144 个氨基酸。氨基酸序列分析表明，该基因与其他物种的 Hb 具有较高的序列相似性。实时定量 PCR 检测显示 CsHB-α1 在心、肝、脾脏、肾脏和血液中表达量较高。与对照组相比（溶解氧：6.2 mg/L），在短期（5 ～120 min）及 36 h 低氧胁迫（溶解氧：1.0 mg/L）后，CsHb-α1 在心、肝脏、脾脏、肾脏、血液和腮腺中表达量明显升高。这表明 Hb-α1 的上调是半滑舌鳎适应低氧胁迫的一个重要分子组成。

关键词：半滑舌鳎；血红蛋白；基因克隆；低氧胁迫
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The increased industrialization and urbanization of coastal zones means that aquatic hypoxia has become a complex ecological phenomenon and now receives much attention (Wawrowski et al, 2010; Gray et al, 2002). Aquatic hypoxia can cause metabolic changes and influence fetal growth and development in all organisms living in water (Kajimura et al, 2004). Fish are the main group of animals affected by hypoxia and it is necessary

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to investigate hypoxia tolerance strategies at the organism level (Roesner et al, 2008).

Hemoglobin (Hb) is used for transporting oxygen in red blood cells and its role in hypoxia has been characterized in several teleosts (Wetten et al, 2010). The expression of mammalian Hb consistently increases at altitude, but data on piscine Hb under hypoxia is variable and follows a species-specific expression pattern. Hb mRNAs were down-regulated in zebrafish (*Danio rerio*) and remained constant in goldfish (*Carassius auratus*) (Ton et al, 2003; Roesner et al, 2006, 2008). In sailfin molly (*Poecilia latipinna*) and Japanese medaka (*Oryzias latipes*) Hb is up-regulated during hypoxia (Timmerman & Chapman, 2004; Wawrowski et al, 2011). The exact role of piscine Hbs under hypoxia is controversial and information from more species is needed.

The half-smooth tongue sole (*Cynoglossus semilaevis*) is a benthic marine fish and an important species in China (Shao et al, 2010). Due to its specific habitat, the half-smooth tongue sole may possess particular adaptations to hypoxia; however, the response of half-smooth tongue sole to hypoxia remains unclear. In this study we cloned and characterized Hb-α1 orthologue in half-smooth tongue sole and examined its expression patterns in response to short-term of hypoxia exposure.

1 Materials and Methods

1.1 Experimental fish

Adult half-smooth tongue sole "mean length: (24.64±1.01) cm; weight: (77.44±8.21) g" were purchased from a local hatchery and kept for two weeks in a 40 L tank with seawater in our laboratory. During the experimental period, the fish were fed twice daily on a 14 h light/ 10 h dark cycle at 21 °C.

1.2 Hypoxia treatment

Groups of five fish were randomly assigned to a hypoxia treatment or control group. Control animals were kept under normoxic dissolved oxygen (DO, 6.2 mg/L), whereas the air stones in hypoxia stress groups were removed and bubbled with nitrogen gas to obtain hypoxic DO levels of 1.0 mg/L. A YSI multiprobe (YSI 556 Multiprobe system) was used to monitor DO, pH and temperature. The heart, liver, spleen, kidney, intestine, stomach, blood and gills from three fish in each group were sampled after continuous exposure for 5, 30, 60, 90 and 120 min, and 36 h, and stored at -80 °C until use.

1.3 RNA extraction and reverse transcription

Total RNA was extracted from the liver using TRIzol Reagen (Invitrogen, USA) according to the manufacturer's instructions. To avoid contamination of genomic DNA, RNA samples were digested with RNase-free DNase I (Takara) incubation for 30 min at 37 °C. First-strand cDNA was synthesized using SuperScript Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction.

1.4 Cloning of CsHB-α1 fragment with RT-PCR and full length with rapid amplification of cDNA ends (RACE)

To obtain the partial sequence of Hb-α1, degenerate primers were designed using Primer 5.0 according to conserved regions of Hb-α1 from human (GenBank accession No. NP_00549), mouse (GenBank accession No. NP_032244), Atlantic cod (*Gadus morhua*) (GenBank accession No. ABV21500), zebrafish (GenBank accession No. AAB05404) and Japanese ricefish (*Oryzias latipes*) (GenBank accession No.BAC20301). The predicted product size was 300 bp. PCR amplification was carried out in a total volume of 25 μL containing 1 μLcDNA template, 2.5 μL 10×buffer, 2 μL dNTPs (TaKaRa), 0.1 μL ExTaq (TaKaRa), 1 μL of each primer and 17.4 μL ddH_2O. The PCR reaction was performed with 1 cycle of 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72°C for 30 s, followed by 1 cycle of 72 °C for 10 min. The amplified fragments were gel-extracted, purified using a TaKaRa agarose gel purification kit, ligated into pMD18-T (TaKaRa) and sequenced on an automatic DNA sequencer (ABI Applied Biosystems Mode 377).

Based on the obtained partial sequence, 3′-RACE and 5′-RACE were performed using the 3′-RACE and 5′-RACE Kit (TaKaRa) to obtain the completed sequence of Hb. The PCR cycling program was 1 cycle of 94 °C for 5 min, 9 cycles of 94 °C for 30 s, 64 °C for 30 s and 72 °C for 90 s, 29 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 90 s, followed by 1 cycle of 72 °C for 10 min. The products were purified and sequenced as mentioned above. All primers used for this analysis are listed in Tab. 1.

1.5 Sequence analysis

The BLAST program was used to identify homologous sequences in the GenBank database. The deduced amino acids were predicted using software on the ExPASy molecular biology server (http://www.

Tab. 1 Primers used for cloning and expression analysis of CsHb-α1

Name	Sequences (5′-3′)	Length	Useage
UPM	*CTAATACGACTCACTATAGGGC*	17	Gene cloning
Hbα1-5out	*CCTGCCCAGAGCCTCTGCGCCG*	22	Gene cloning
Hbα1-5in	*GCTCCAGAGGATCCTGATGCGC*	22	Gene cloning
Hbα1-3out	*CCAACGACTACACCGACGAGG*	21	Gene cloning
Hbα1-3in	*GGCCCACCTCTCCTTCGACAA*	21	Gene cloning
Hbα1-F1	*AACCAAGACCTACTCCACCCA*	21	Cloning and expression
Hbα1-R1	*CTTCTCGCTGAGGTCCAACA*	20	Cloning and expression
actin-F	*CAGCCATACTGTGCCCATCT*	20	Expression
actin-R	*TCCTTGATGTCACGCACGAT*	20	Expression

expasy.org). Multiple sequence alignments were generated by CLUSTAL 1.8 (Clewley & Arnold, 1997). Identities between amino acids were determined using MegAlign in DNAStar. Conserved domains of protein were analyzed using PROSITE, available on the ExPASy server (http://www.expasy.org).

1.6 Quantitative real-time PCR

To examine basal and hypoxic expression, eight tissues (heart, liver, spleen, kidney, intestine, stomach, blood and gill) were collected from three *C. semilaevis* reared under normoxic and undere hypoxia. After total RNA extraction and DNase treatment, 2 μg of total RNA was transcribed into cDNA using PrimeScript® RT reagent kit (TaKaRa). All cDNA samples were stored at −20 °C until use.

C. semilaevis β-actin (reference gene) (GenBank accession No. GH234955) and Hb-α1 fragments were amplified by RT-PCR and subcloned into pMD18-T vector and confirmed by sequencing. The plasmid DNA was extracted with a QIAGEN plasmid mini kit and the concentration was measured by spectrophotometer at OD_{260}. The corresponding copy number was calculated as 1μg of 1 000 bp DNA=9.1×10^{11} molecules. Serial ten-fold dilutions of the plasmid, ranging from 10^8 to 10^2 input cDNA copies, were used as a standard curve in the PCR run. RT-PCR experiments were performed on an ABI RT-PCR system (Applied Biosystems). Amplifications were carried out at a final volume of 20 μL, containing 1 μL cDNA template, 10 μL SYBR premix ExTaq, 0.4 μL 50×Rox reference dye, 1 μL of each primer and 6.6 μL ddH_2O. The PCR amplification were performed in triplicate wells and carried out using a two-step method: 30 s at 95 °C, followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Specificity of amplification was analyzed using dissociation curves with temperature ranging from 60 − 95 °C. The molecular number of a particular gene transcript was calculated based on the standard curve and normalized to the β-actin level (Shen et al, 2010). Gene expression in normal tissue was measured relative to the gene expression of β-actin (Qi & Nie, 2008). The ratio target gene expression after hypoxic exposure were expressed as fold changes and calculated using the formula: folds = fold change in target gene expression (experimental group/control group)/fold change in reference gene expression (experimental group/control group) (Qi et al, 2011).

Data were retrieved from the ABI Prism 7300 SDS program and analyzed with Microsoft Excel. Results are expressed as Mean±*SE*. Statistical significance was evaluated using student's *t*-tests.

2 Results

2.1 Cloning and analyses of CsHb-α1 gene

By using RT-PCR and RACE methods, the full-length of CsHb-α1 gene was obtained and submitted to the GenBank database (accession No. HQ219034). CsHb-α1 was 594 bp in length, with a 41 bp 5′ untranslated region (UTR), a 432 bp open reading frame (ORF) coding 143 aa, and a 121 bp 3′ UTR. One polyadenylation signal (aataaa) was found followed by a poly (A) tail in the 3′ UTR (Fig.1).

The CsHb-α1 was aligned with other Hb-α1 proteins retrieved from GenBank. The CsHb-α1 shared 46.2% − 58% identities with other Hb-α1s. The lowest sequence identity was 46.2% with human Hb-α1 and the highest was 58% with Japanese ricefish HB-α1. CsHb-α1 also contains six conserved α helices (designated as A-H). A comparison of the deduced amino acid sequence of CsHb-α1 and human Hb-α revealed 7 of 15 α1-β1 interface, 12 of 13 α1-β2 interface and 3 of 6 α-hemoglobin stabilizing protein (α-AHSP) interacting sites were similar (Yu et al, 2009) (Fig.2).

```
   1 ggggaattccagttgttagcagcacaggaaccgaagccaaaATGAGTCTGAACAGCATTG
   1                                          M  S  L  N  S  I  D
  61 ACAAGGAGCGCATCAGGATCCTCTGGAGCAAAATCTCCAAAGACTCTGACGCCATCGGCG
   8  K  E  R  I  R  I  L  W  S  K  I  S  K  D  S  D  A  I  G  A
 121 CAGAGGCTCTGGGCAGGTTGTTCGCGGCGCACCCCCAAACCAAGACCTACTCCACCCACT
  28  E  A  L  G  R  L  F  A  A  H  P  Q  T  K  T  Y  S  T  H  F
 181 TTAAGGATTTTACTTACAACAGCCCTCAGGTGAAGGAGCATGGGAAGCTGGTGATGAAAG
  48  K  D  F  T  Y  N  S  P  Q  V  K  E  H  G  K  L  V  M  K  G
 241 GAATCAAGCAGGCCTACGAGAACATCGACGACATGGTCACCGGGCTGTTGGACCTCAGCG
  68  I  K  Q  A  Y  E  N  I  D  D  M  V  T  G  L  L  D  L  S  E
 301 AGAAGCACGCCTTCACCCTGAGAGTGGATCCCAGCAACTTCAAGTTGCTGTCTAGTTGCT
  88  K  H  A  F  T  L  R  V  D  P  S  N  F  K  L  L  S  S  C  F
 361 TCCACGTGGTCCTTTCCAAGAGGTACCCCAACGACTACACCGACGAGGCCCACCTCTCCT
 108  H  V  V  L  S  K  R  Y  P  N  D  Y  T  D  E  A  H  L  S  F
 421 TCGACAAATTCCTTGCCAACGTGGCTCTGGCTCTGTCCGAGAAATACCGCTAAactgcac
 128  D  K  F  L  A  N  V  A  L  A  L  S  E  K  Y  R  *
 481 ccagtcagcgacaggcatattggcatagcgccaccacatggacgctgccgaaaaatgtca
 541 aatataagaagatacaaataaacttcacattaactaaaaaaaaaaaaaaaaaaa
```

Fig.1 cDNA sequences of CsHb-α1 (GenBank accession No. HQ219034) and deduced amino acids

The stop codon (TAA) is marked as asterisk (*). Polyadenylation signal (aataaa) underlined.

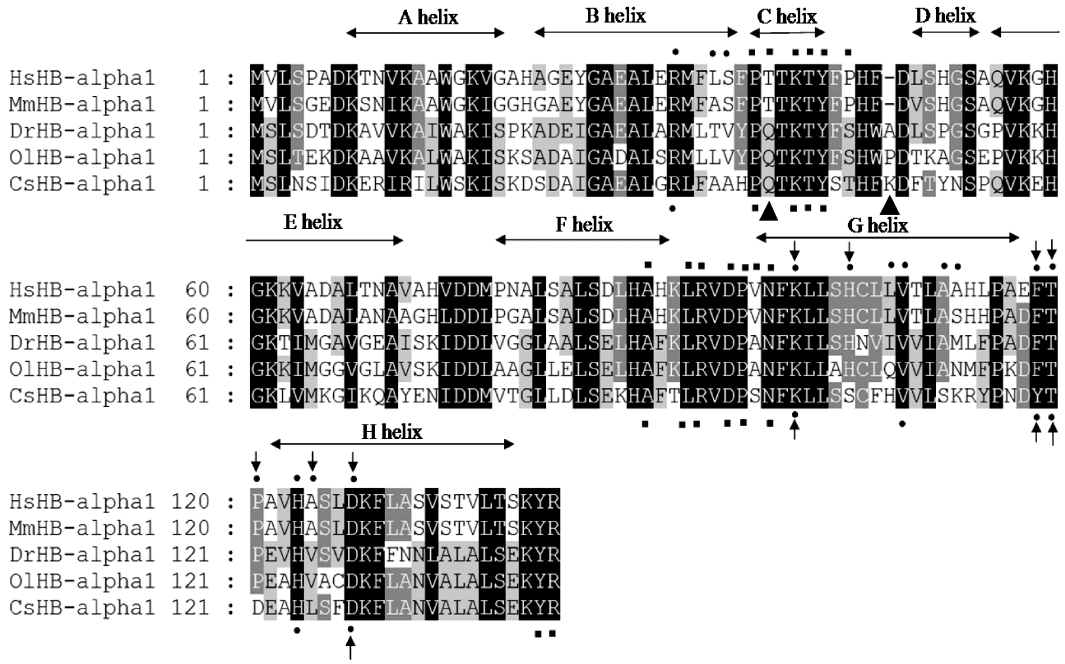

Fig.2 Alignment of deduced *Cynoglossus semilaevis* Hb-α1 amino acid (CsHB-α1) sequence with Hb-α1 homologues from human (HsHB-α1), mouse (MmHB-α1), zebrafish (DrHB-α1), and Japanese ricefish (OlHB-α1)

The conserved α helices are marked as double arrow above the sequence comparisons. The triangle arrow above a sequence comparison shows the predicted AHSP-binding sites of human Hb-α1. Circles above a sequence show residues that come into close contact with β globin at α1β1 interface in human Hb-α1. Squares above a sequence show β globin contact sites at the α1β2 interface of human Hb-α1. The symbols below the sequence comparisons show the conserved amino acids in CsHb-α1. Changes at amino acids sites are marked as triangles below sequence comparisons.

2.2 Basal expression of CsHb-α1

RT-PCR was used to determine gene expression and tissue distribution of Hb-α1. As shown in Fig.3, CsHb-α1 was detected in all examined tissues. It was

abundantly expressed in the heart, liver, spleen, kidney and blood, weakly in the stomach and intestine, and moderately in the gills.

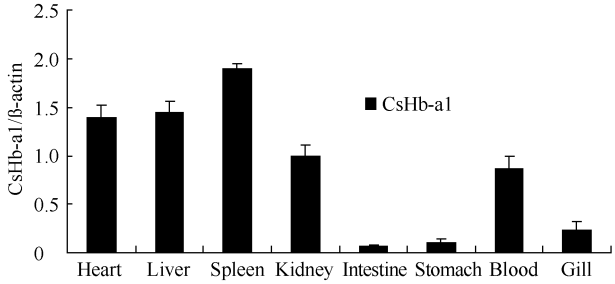

Fig.3 Basal expression of CsHb-α1 in tissues of normal *Cynoglossus semilaevis*

The expression of CsHb-α1 was detected using real-time PCR and normalized with the expression of β-actin. n=3.

2.3 CsHb-α1 expression under hypoxia

RT-PCR was employed to determine the changes in CsHb-α1 mRNA levels in different tissues after hypoxia exposure (DO=1.0 mg/L). Under hypoxia for 5–120 min, the expression of CsHb-α1 was significantly increased in the heart, liver, spleen, kidney, blood and gills. Meanwhile, the expression of CsHb-α1 in the intestine was increased only at 5 min, and decreased from 30–120 min. After 36 h of hypoxia exposure, CsHB-α1 was up-regulated greatly in most tissues with 21.8 fold changes in the heart, 22.3 fold changed in the blood, 12 fold changes in the spleen, 11.5 fold changed in the kidney and 21.2 fold changes in the gills. There were no significantly changes in the stomach after hypoxia (Fig.4).

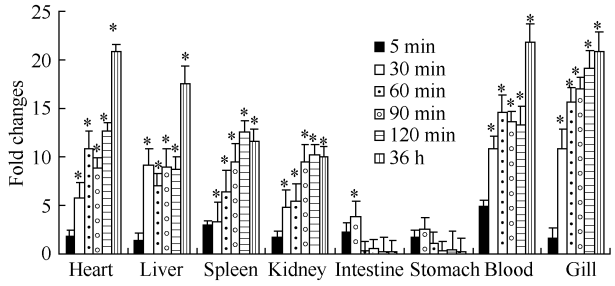

Fig.4 Relative expression of CsHb-α1 under hypoxia for 5–120 min and 36 h in different tissues of *Cynoglossus semilaevis*

The ratio of target gene expression was calculated using the formula: folds=fold change in target gene expression (experimental group/control group)/ fold change in reference gene expression (experimental group/control group). Bars represent standard errors of the means. n=3. *: P<0.05.

3 Discussion

In this study the Hb-α1 gene was cloned and characterized in *Cynoglossus semilaevis* and the effect of short-term hypoxia on gene expression was examined. Sequence analysis showed that CsHb-α1 possessed six α helical structure and this multi-helix-structure was conserved during evolution. The obtained CsHb-α1 was 143 aa in length and this is consistent for piscine Hb-α1 (Chan et al, 1997). Some important functional amino acids sites for human Hb-α1 were also found in CsHb-α1, such as amino acids at the α1β1 and α1β2 interface. This evidence confirms that the gene we cloned was an exact Hb-α1 homologue.

Changes at some amino acids sites were also observed in Hb-α1: an amino acid insertion at position 47 of piscine Hb-α1 with Ala in zebrafish Hb-α1, Pro in Japanese ricefish and Lys in CsHb-α1 (Fig.2). The amino acid at position 47 of mammalian Hb-α1 was deleted. Miyata et al (1993) suggested that an insertion of residue at position 47 in carp α-globin produced only inconsequential changes in function and the structure of globin. There was a change at position 39 (Thr to Gln), and this change was also observed in CsHb-α1 (Fig.2) and other teleost and turtle α-globin (Petruzzelli et al, 1996). The Thr 39 is considered to play a crucial role in forming the hydrogen-bond between Hb-β and Hb-α. However, recent results of mutation of the Thr 39 by serine or valine showed that this residue may not play an essential role in stabilizing the deoxy T structure (Hashimoto et al, 1993). Analyses to date suggest that changes in amino acids in Hb-α1 during evolution mainly occur at positions that do not affect the function or structure of Hb-α1.

The expression of Hb-α1 has been studied in several teleosts and piscine Hb-α1 is constitutively expressed in most tissues. Our study shows that CsHb-α1 is expressed in most tissues including the heart, liver, spleen, kidney and blood, similar to observations in zebrafish and other teleosts (Ton et al, 2003; Roesner et al, 2008; Wawrowski et al, 2010). Unlike humans, teleosts do not have bone marrow and the haemopoietic tissue of fish is found mainly in the liver, kidney and spleen (Willet et al, 1999). This may explain the specific expression pattern of piscine Hb-α1.

Although Hb had been characterized from several teleost fishes, the expression pattern of piscine Hb under hypoxia differs between species. In Sailfin molly, an increase in Hb was detected in blood under chronic hypoxia stress (six weeks at DO=1.0 mg/L). Wawrowski et al (2011) also observed an increase in Hb mRNA in

the brain and whole fish of Japanese medaka. While in zebrafish, down-regulation of embryonic Hb mRNA was observed in embryos (Ton et al, 2003) and reduced levels of adult Hb was detected in whole adult fish (van der Meer et al, 2005; Roesner et al, 2006). Roesner et al (2008) found that goldfish Hb remained constant in carcasses. Therefore, the hypoxia response of Hb in fish may be species-specific (Wawrowski et al, 2011). To further understand the response of piscine Hb under hypoxia, we selected the marine fish half smooth tongue sole as an experimental model and examined expression of Hb-α1 under short-term hypoxia. We found that the CsHb-α1 was up-regulated in haemopoietic tissue such as the liver, spleen, and kidney during short-term hypoxia. This increase in CsHb-α1 was also observed at extended hypoxia of 36 h. This finding suggests the fish activates haematopoiesis process to raise Hb levels, increase the O_2-transport capacity of blood and enhance oxygen supply to the inner organs during hypoxic stress (Wawrowski et al, 2010). That CsHb-α1 in the stomach and intestine remained unchanged may be because the fish requires more Hb to transport O_2 to relatively important tissues (liver and heart) to maintain normal physiological activity under short-term hypoxia and to save energy may reduce Hb levels in peptic tissues.

Hb-α1 was cloned and characterized from half-smooth tongue sole. CsHb-α1 shared high identities with Hb-α1 from other teleosts and some functional motifs and amino acids sites were conserved. Under short term hypoxia, CsHb-α1 was up-regulated in most tissues except peptic ones. This up-regulation of Hb-α1 may be an important component in the adaptation of half-smooth tongue sole to short-term hypoxia.

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